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Interleukin-13-induced MUC5AC expression is regulated by a PI3K–NFAT3 pathway in mouse tracheal epithelial cells

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ABSTRACT

Interleukin-13 (IL-13) plays a critical role in asthma mucus overproduction, while the mechanisms underlying this process are not fully elucidated. Previous studies showed that nuclear factor of activated T cells (NFAT) is involved in the pathogenesis of asthma, but whether it can directly regulate IL-13-induced mucus (particularly MUC5AC) production is still not clear. Here we showed that IL-13 specifically induced NFAT3 activation through promoting its dephosphorylation in air–liquid interface (ALI) cultures of mouse tracheal epithelial cells (mTECs). Furthermore, both Cyclosporin A (CsA, a specific NFAT inhibitor) and LY294002 (a Phosphoinositide 3-kinase (PI3K) inhibitor) significantly blocked IL-13-induced MUC5AC mRNA and protein production through the inhibition of NFAT3 activity. We also confirmed that CsA could not influence the forkhead Box A2 (Foxa2) and mouse calcium dependent chloride channel 3 (mClca3) expression in IL-13-induced MUC5AC production, which both are known to be important in IL-13-stimulated mucus expression. Our study is the first to demonstrate that the PI3K–NFAT3 pathway is positively involved in IL-13-induced mucus production, and provided novel insights into the molecular mechanism of asthma mucus hypersecretion.

1. Introduction

Airway mucin hypersecretion (particularly MUC5AC and MUC5B) contributes to morbidity and mortality in asthma [1]. Previous studies have demonstrated that interleukin-13 (IL-13) plays a critical role via IL-13 receptor and STAT6 transcription factor [2,3] in asthma mucus overproduction [4–6], while the mechanisms underlying this process still need to be defined.

Nuclear factor of activated T cells (NFAT) is a transcription factor originally identified in T cells, composed of four distinct members that are regulated by the calcium/calcineurin signaling pathway, known as NFAT1 (also called NFATp, NFATc2), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx, NFATc3). Another NFAT member called NFAT5 (TonE-BP) is regulated by hyperosmotic stress. NFAT has been confirmed to be involved in the pathogenesis of asthma [7–9]. Fonseca et al. [8] showed a significant decreased number of mucus-producing cells in the lung of OVA-challenged NFAT1$^{-/-}$ mice, but whether or not NFAT can directly regulate mucus production is still not clear. On the other hand, several transcription factors including forkhead Box A2 (Foxa2) and SAM pointed domain-containing ETS transcription factor (SPDEF) which are involved in lung morphogenesis and respiratory epithelial differentiation play an important role in asthma mucus overproduction [10–11], and Davé et al. have shown that NFAT is also required for perinatal lung maturation and function [12]. Therefore, we hypothesize that NFAT may also participate in regulation of asthma MUC5AC expression.

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that mediates various cellular functions, including mitogenesis, survival, motility, and differentiation. PI3K is involved in IL-13-induced increase of goblet cells density [13], and can induce NFAT activation in T cells [14]. Furthermore, Moon et al. [15] showed PI3K is involved in osteoclast differentiation through NFAT2, so we questioned whether a PI3K–NFAT cascade mediated IL-13-induced MUC5AC expression.

As mentioned, Foxa2 has a crucial role in IL-13-induced MUC5AC expression [10], and NFAT4 is a direct activator of Foxa2 genes [12], so we further explored whether NFAT is dependent on foxa2 to function its role. Calcium dependent chloride channels (CLCA) of airway epithelial cells play an important role in the regulation of mucus production, and mouse calcium dependent chloride channel 3 (mClca3, the homolog of human CLCA1) can

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mediate IL-13-induced mucus overproduction [16], we therefore sought to investigate the correlation between NFAT and mClca3 in IL-13-induced MUC5AC expression.

Here we firstly demonstrated that a PI3K–NFAT3 pathway is positively involved in IL-13-induced MUC5AC expression in air–liquid interface (ALI) cultures of mouse tracheal epithelial cells (mTECs), and Cyclosporin A (CsA) could not influence the Foxa2 and mClca3 expressions in IL-13-induced MUC5AC production. These findings may provide new insights into the pathogenesis of asthma mucus overproduction.

2. Materials and methods

2.1. Materials

Recombinant human IL-13 was purchased from R&D, Inc. CsA-was from Sigma, Inc. Antibodies against MUC5AC (45M1), mClca3, Foxa2, p-NFAT1 (Ser274) and p-NFAT2 (Ser259) were from Santa Cruz, Inc. Antibodies against p-NFAT3 (phosphor S168 + S170), p-NFAT4 (phosphor S165), and β-actin were from Abcam, Inc. LY294002 was from Calbiochem, Inc.

2.2. Primay mouse tracheal epithelial cells culture

Primary air–liquid interface cultures of mTECs were established as described previously [17]. Mouse trachea was isolated from 8 to 10 week-old C57BL/6 mice under sterile conditions, and digested with 10 mL 0.15% pronase solution overnight at 4 °C. Then tracheal epithelial cells were harvested and submerged-cultured with mTECS proliferation medium (DMEM/F12 basic media add HEPES, glutamine solution, NaHCO3, heat-inactivated FBS, Retinoic acid, Insulin, Epidermal growth factor solution, bovine pituitary extract, Transferrin) in transwell plates (Corning, NY) for 10–14 days. When cells were confluent, the medium in apical side was removed and ALI culture began. After 1-week of ALI culture, cells were stimulated by adding IL-13 (10 ng/mL) alone or in combination with CsA or LY294002 in the basal wells for 14 days, and the medium was replaced every other day.

2.3. Real-time PCR

Total RNA was isolated from mTECs with ALI cultures using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. For RT-PCR, cDNA was generated by reverse transcription using 2 μg total RNA. The expression levels of MUC5AC, NFAT1, NFAT2, NFAT3, NFAT4, mClca3, and Foxa2 mRNA were determined by quantitative real-time PCR using the SYBR Green system (Takara) on a spectrofluorometric thermal cycler (iCycler; Bio-Rad). The PCR primers are as follows: mouse MUC5AC: forward, GGACTTCAATATCCAGCTACGC, reverse, GGACTTCAATATCC AGCTACGC; mClca3: forward, ACTAAGGTGGCCTACCTC CAA, reverse, GGAGGTGACAGTCAAGGTGAGA; mouse NFAT1: forward, C CACCACGAGCTATGAGAAGA, reverse, GTCAGCGTTTCGGAGCTTCA ; mouse NFAT2: forward, CAGTGTGA CCGAAGATACCTG G, reverse, TCGAGACTTGATA GGGACCCC; mouse NFAT3: forward, GAGCTGGAATTTAAGCTGGT GT, reverse, CATGGA GGGGTATCCTCTGAG; mouse NFAT4: forward, GTATGGATCTGG ACACTCCTTGT, reverse, CGTCGTTTACCACAGGGAGA; mouse Foxa2: forward, CAT GGGACCTCACCTGAGTC, reverse, CATCGAGT TCATGTT GGCG TA; mouse β-actin: forward, GGCTGTATTCCCCTCCATCG, reverse, CCAGTTGGTAACAA TGCCATGT.

2.4. Western blotting analysis

After stimulation for 14 days, the cells were harvested in cell lysis buffer, separated on 8–10% SDS–PAGE gels and transferred to polyvinylidine difluoride membranes (Pall Life Sciences, Pensacola, FL). The membrane was blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% nonfat milk. After three washes in TBS-T, the membrane was incubated with a 1:1000 dilution of a primary antibody for overnight. After another three washes in TBS-T, the membrane was incubated with 1:2000 dilution of the corresponding secondary antibody for 1 h. The membrane was reacted with enhanced chemiluminescence (ECL, Amersham Biosciences) to visualize to blots.

2.5. Statistical analysis

Data are presented as mean ± SD ($n = 3$). ANOVA was used to determine statistically significant differences ($P < 0.05$).

3. Results

3.1. IL-13 induced NFAT3 activation and MUC5AC expression in mTECs

Consistent with previous reports [19], we also demonstrated that the expression of MUC5AC mRNA and protein were significantly increased in ALI cultures of mTECs treated with IL-13 (10 ng/mL) for 14 days (Fig. 1A and B), while only mildly increased in those stimulated by 1 ng/mL of IL-13 for 24 h, 48 h and 14 days as well as 10 ng/mL of IL-13 for 24 h and 48 h respectively. To determine the role of NFAT in IL-13-induced MUC5AC expression, we analyzed NFAT1, NFAT2, NFAT3 and NFAT4 mRNA expression in the ALI cultures of mTECs treated with IL-13 (10 ng/mL) for 24 h, 48 h and 14 days respectively. However, we found no significant up-regulation of all four NFAT mRNA levels (data not shown), which indicated that IL-13 may induce the activation of NFAT proteins and not their expression levels. Next we explored the activation and expression levels of NFAT1, NFAT2, NFAT3 and NFAT4 proteins. The activation process of NFAT is as follows: extra

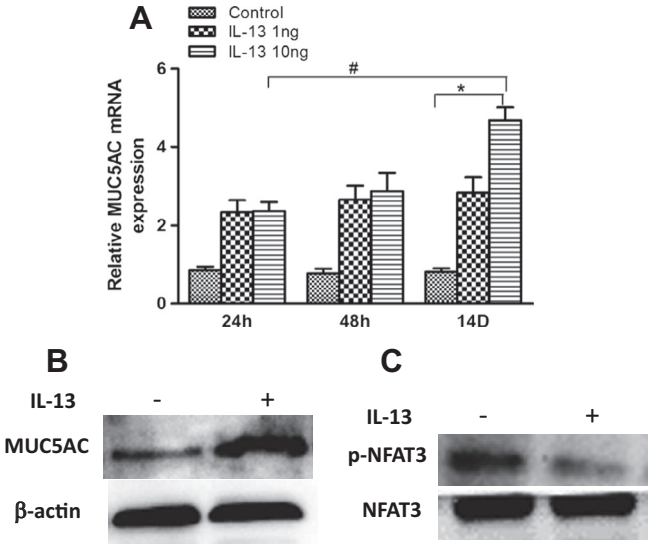

Fig. 1. IL-13 induced NFAT3 activation and MUC5AC expression in mTECs. (A) The expression of MUC5AC mRNA in ALI cultures of mTECs treated with IL-13 (1 ng/mL, 10 ng/mL) for 24 h, 48 h and 14 days. (B) The MUC5AC protein expression in ALI cultures of mTECs treated with IL-13 (10 ng/mL) for 14 days. (C) The phosphorylated NFAT3 and total NFAT3 levels in mTECs treated with IL-13 (10 ng/mL) for 14 days. Data are expressed as means ± SD ($n = 3$). $^{\#}P < 0.05$, $^{*}P < 0.01$.

stimulation leads to an increased intracellular Ca^{2+}, then the calcineurin enzyme becomes active and dephosphorylates NFAT, allowing NFAT translocation into the nucleus and subsequent regulation of gene expression [18]. After treated with IL-13 (10 ng/mL) for 14 days, we found that only the phosphorylated NFAT3 was obviously decreased in mTECs, but the total NFAT3 protein level was not changed (Fig. 1C). These findings suggested that IL-13 did not up-regulate NFAT1, NFAT2, NFAT3 and NFAT4 mRNA and total proteins expression, but it could specifically induce NFAT3 activation through promoting its dephosphorylation in airway epithelial cells.

3.2. NFAT3 was positively involved in IL-13-induced MUC5AC expression

To clarify the role of NFAT3 in IL-13-induced mucus production, we utilized different dose of CsA (50 nM, 100 nM, 200 nM) which is known to inhibit the function of NFAT [20] to stimulate mTECs alone or in combination with IL-13 (10 ng/mL) for 14 days, as genetic approaches are difficult to be used in primary epithelial cultures. As shown in Fig. 2A and B, CsA (100 nM) significantly inhibited IL-13-induced MUC5AC mRNA and proteins expression in mTECs. Furthermore, CsA obviously inhibited IL-13-induced NFAT3 proteins activation (Fig. 2C), suggesting NFAT3 is positively involved IL-13-induced MUC5AC expression.

3.3. PI3K was positively involved IL-13-induced MUC5AC through NFAT3

To investigate whether PI3K mediated IL-13-induced MUC5AC expression through NFAT3, We used specific PI3K inhibitor LY294002 (2 μM) to treat mTECs alone or in combination with IL-13 (10 ng/mL) for 14 days. As shown in Fig. 2A–C, LY294002 strikingly inhibited IL-13-indued MUC5AC mRNA and protein expression, as well as NFAT3 protein activation. These results suggested that PI3K was positively involved IL-13-induced MUC5AC expression through NFAT3 in airway epithelial cells.

3.4. CsA could not influence the Foxa2 and mClca3 expression in IL-13-induced MUC5AC production

We have shown a PI3K–NFAT3 pathway was positively involved IL-13-indued MUC5AC production (Fig. 4), but whether or not this effect was dependent on Foxa2 and mClca3, which both are known to play an important role in IL-13-induced mucus over-production, is still not clear. As shown in Fig. 3A and B, IL-13 (10 ng/mL) significantly inhibited Foxa2 mRNA and proteins expression in mTECs, which could not be influenced by CsA. Furthermore, IL-13 strikingly up-regulated mClca3 mRNA and proteins expression, but which could not be inhibited by CsA (Fig. 3C and D). These findings suggested Foxa2 and mClca3 were not the downstream signaling events of NFAT3 in IL-13-induced MUC5AC expression.

4. Discussion

Our study is the first to demonstrate that a PI3K–NFAT3 pathway is positively involved in IL-13 induced mucus production. We showed that IL-13 specifically induced NFAT3 protein activation in mTECs, both a specific NFAT inhibitor CsA and PI3K inhibitor LY294002 significantly inhibited IL-13-induced MUC5AC mRNA and proteins production through the inhibition of NFAT3 activity. We also suggested that CsA could not influence the Foxa2 and mClca3 expression in IL-13-induced MUC5AC production.

Previous numerous studies of NFAT mainly focused on its function in immune cells [21]. Here we elucidated the role of NFAT3 in IL-13-induced mucus production in airway epithelial cells which provide evidence of NFAT function in non-immune cells. Of course, though we have confirmed the role of NFAT3 in vitro, whether NFAT3 plays the same role for mucus overproduction in vivo needs to be further investigated.

Transcription factors of the NFAT family play key roles in the pathogenesis of asthma. Diehl et al. [9] demonstrated that activation of NFAT in CD4 T cells is required for the development of a Th2 immune response in vivo and allergic airway inflammation. Fonseca et al. [8] showed OVA-challenged $NFAT^{-/-}$ mice developed

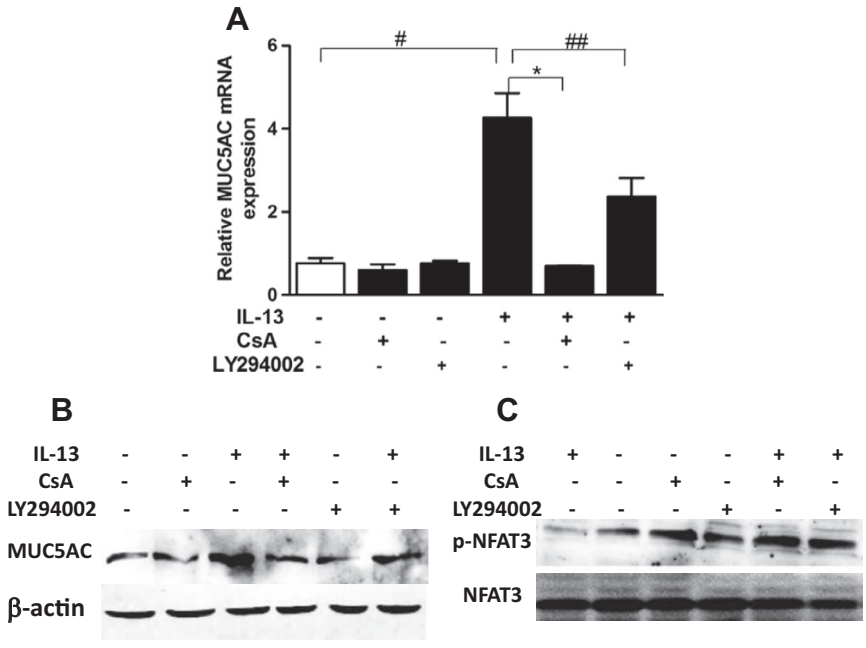

Fig. 2. The PI3K–NFAT3 pathway was positively involved in IL-13-induced MUC5AC expression. (A, B) The MUC5AC mRNA and protein expression in mTECs treated with CsA (100 nM) and LY294002 (2 μM) alone or in combination with IL-13 (10 ng/mL) for 14 days. (C) The phosphorylated NFAT3 and total NFAT3 levels in mTECs treated with CsA (100 nM) and LY294002 (2 μM) alone or in combination with IL-13 (10 ng/mL) for 14 days. Data are expressed as means ± SD ($n = 3$). $^{\#}P < 0.01$, $^{\#\#}P < 0.05$, $^{*}P < 0.01$.

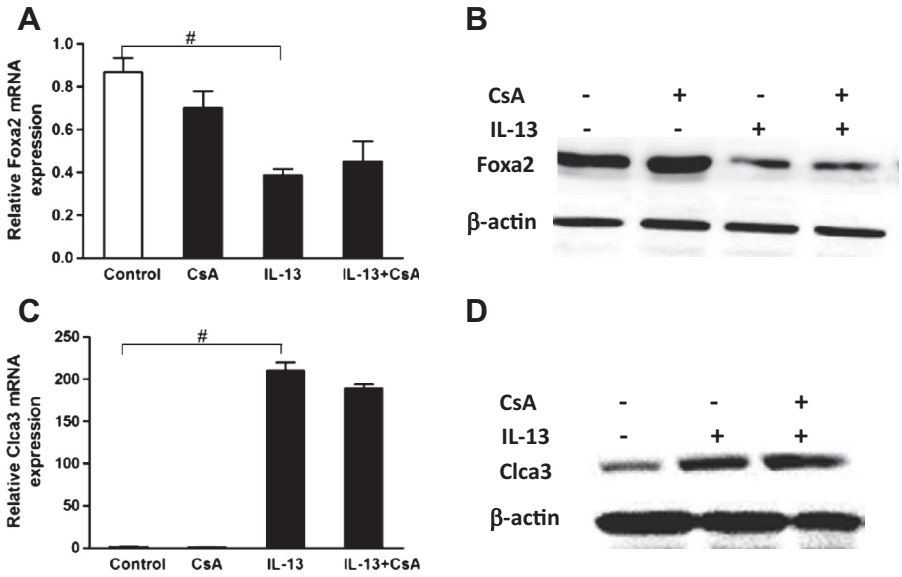

Fig. 3. CsA could not influence the Foxa2 and mClca3 expression in IL-13-induced MUC5AC production. (A, B) The Foxa2 mRNA and protein levels in mTECs treated with CsA (100 nM) alone or in combination with IL-13 (10 ng/mL) for 14 days. (C, D) The mClca3 mRNA and protein levels in mTECs treated with CsA (100 nM) alone or in combination with IL-13 (10 ng/mL) for 14 days. Data are expressed as means ± SD ($n = 3$). $^{\#}P < 0.01$.

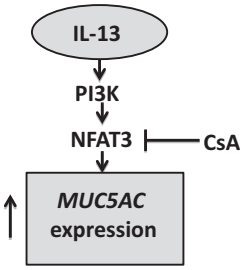

Fig. 4. Schematic representation depicting how NFAT3 mediates IL-13-induced MUC5AC expression in airway epithelial cells.

a significant exacerbation of lung inflammation, eosinophilia, and serum IgE levels, while marked remission of airway hyperresponsiveness (AHR) and mucus secretion. However, Fonseca did not answer whether or not NFAT directly regulated mucus overproduction of asthma. Here we firstly provided the direct evidence that NFAT3 is involved in IL-13-induced mucus overproduction. On the other hand, we confirmed that it is not NFAT1, but NFAT3 to mediate IL-13-induced mucus expression, which suggested NFAT1 may inhibit asthma mucus overproduction via other indirect pathway. Both Foxa2 and mClca3 play key roles in IL-13 driven mucus production [10,16], and Davé et al. showed NFAT4 was a direct activator of foxa2 gene [12]. However, we found CsA could not influence the Foxa2 and mClca3 expression in IL-13-induced MUC5AC production, so the downstream signaling events of NFAT3 and whether NFAT3 itself can directly regulates promoter activity of MUC5AC in IL-13-induced mucus production needed to be further explored.

There are many studies involved the indispensible role of PI3K in mucins regulation [22–24]. Song et al. showed induction of MUC5AC mucin by conjugated bile acids in the esophagus involves the PI3K/activator protein-1 (AP-1) pathway [22]. Binker et al. demonstrated a PI3K/Rac1/NADPH cascade regulate MUC5AC production in LPS-challenged NCI-H292 cells[23]. In terms of IL-13-induced mucus secretion, Atherton et al. [13] showed that IL-13-driven increase in MUC5AC protein expression involves an LY-294002-sensitive pathway, while the detailed mechanisms underlying this is not clear. Another report demonstrated that PI3K is positively involves in IL-13-induced MUC5AC expression through inhibiting the apoptosis of ciliated airway epithelial cells [25]. In the present study we confirmed PI3K plays an important role in IL-13-induced MUC5AC expression dependent on NFAT3, and suggested a PI3K–NFAT3 signaling pathway mediates IL-13-induced mucus production. These findings suggested PI3K may regulate IL-13-stimulated mucus expression via different molecular mechanism.

In summary, we, for the first time, confirmed the key roles of the PI3K–NFAT3 pathway in IL-13-induced mucus production, which provided novel insights into the molecular mechanism of asthma mucus hypersecretion.

Acknowledgments

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